GENERAL INTRODUCTION

- Spectrophotometry uses the light-absorbing capacity of a substance to determine how much of the substance is present in a given sample (i.e., the substance concentration)

- Spectrophotometers are instruments that measure the amount of light absorbed by a substance in solution

- This module reviews the use of a particular type of spectrophotometer, the Spectronic 20D+
How the spectrophotometer works: White light — containing all colors of the rainbow—from a lamp inside the instrument is directed at a monochromator which selects a single wavelength of light (i.e., a color) to shine on the sample. The sample—which consists of a substance, a dissolving liquid, and it is contained in a glass cuvette—absorbs some of the light. The remaining light is transmitted to a detector which measures the decreased intensity of the light beam due to sample absorption.
GENERAL INTRODUCTION

- By following the procedures in this module, you will be able to isolate the light-absorption of just your substance of interest and proceed to quantify the exact concentration of that substance in solution.

- More generally, spectrophotometry will allow you to quantify the concentrations of a variety of substances that are often sampled from the environment (e.g., contaminants in air, soil, and water).
PROCEDURE

A. You will first calibrate the Spectronic 20D+
   i. The instrument needs to be warmed up
   ii. The liquid in which your substance of interest is dissolved & the glass cuvette can influence how much light is transmitted to the detector (i.e., how much light is absorbed before it reaches the detector).

   a. The absorbance of the dissolving liquid and the glass cuvette needs to be set to zero before you can measure just the light-absorbance of your substance of interest.
PROCEDURE

B. You will then use the calibrated Spectrophotometer to generate a Spectrogram

i. The Spectrogram is a graph which shows the amount of light absorbed by the substance of interest at a variety of light wavelengths

ii. The Spectrogram is used to determine the wavelength at which light is most strongly absorbed by the substance of interest ($\lambda_{\text{max}}$)
PROCEDURE

C. You will then use the results of your Spectrogram to create a Calibration Curve

i. The Calibration Curve plots the exact concentration of the substance of interest versus the amount of light absorbed by the substance

ii. Keeping the Spec 20D+ wavelength set to $\lambda_{\text{max}}$, create a Calibration Curve based on the absorbances of several samples with known concentrations

D. Finally, use the Calibration Curve to determine the concentration of an Unknown Sample
MODULE PROCEDURE

➢ You will use the Spec 20D+ to measure the amount of light absorbed by the blue colored solutions (CuSO₄ · H₂O) in the cuvettes at the workstations

➢ For the posttest,
  ➢ Submit a properly labeled Spectrogram
  ➢ Submit a properly labeled Calibration Curve
  ➢ And determine the concentration of the unknown solution.
Turn on your Spec 20D+ so that it warms up for 15 minutes before the sample reading

Rotate this dial to the right to turn on the Spec 20D+
Make sure that you have the following before you begin:

- 3 blue “sample solutions” of known concentration in glass cuvettes
- Kimwipes
- Empty glass cuvettes & a test tube rack
- 2 pieces of graph paper
- 1 ruler
- Distilled water in a squeeze bottle
A. SPEC 20D+ CALIBRATION

SUMMARY

• STEP 1: Turn on – Warm up 15 min.
• STEP 2: Set Wavelength
SET WAVELENGTH

- Ensure that the lever in the bottom left corner is in the correct range
- For this experiment, your wavelengths will all be in the 600 – 950nm range
The wavelength selector knob is on the top right side of the instrument.

Start with the wavelength set to 610 nm.
A. SPEC 20D+ CALIBRATION

SUMMARY

• STEP 1: Turn on – Warm up 15 min.
• STEP 2: Set Wavelength
• STEP 3: Set Mode to Transmittance
• STEP 4: Set Transmittance to 0%
Press the Mode Key to bring the instrument to the transmittance mode.
Set Transmittance

- Make sure the sample holder is empty & the cover is closed.
- The transmittance adjust knob is the left dial on the front of the machine.
- Set your transmittance to 0%.
A. SPEC 20D+ CALIBRATION

SUMMARY

• STEP 1: Turn on – Warm up 15 min.
• STEP 2: Set Wavelength
• STEP 3: Set Mode to Transmittance
• STEP 4: Set Transmittance to 0%
• STEP 5: Set Mode to Absorbance
SET MODE TO ABSORBANCE

Press the Mode Key to bring the instrument to the absorbance mode.
A. SPEC 20D+ CALIBRATION

SUMMARY

- STEP 1: Turn on – Warm up 15 min.
- STEP 2: Set Wavelength
- STEP 3: Set Mode to Transmittance
- STEP 4: Set Transmittance to 0%
- STEP 5: Set Mode to Absorbance
- STEP 6: Prepare and Insert Blank
PREPARE BLANK

- In order to finish calibrating the instrument, you will need to create a Blank.

- The Blank should contain all of the solution components except for the light absorbing substance of interest.
  - Your blue colored (CuSO$_4$) solutions are dissolved in distilled water → your Blank will contain just distilled water.
PREPARE BLANK

- In case the cuvette test tube for your Blank is not clean & dry, you should rinse it thoroughly with the solution you will be using to set up the Blank (distilled water).

- Several small rinses are preferred to just one big rinse in order to coat the inside of the cuvette.
PREPARE BLANK

Fill the cuvette with your solution (distilled water) to a sufficient height so that the internal light beam passes through the solution in the cuvette, and not just through air.

Good Blank!

Bad Blank!
PREPARE BLANK

- Errors may occur if air bubbles are present in the solution.
- Before reading any sample (even a Blank), you must remove all air bubbles.
PREPARE BLANK

- Remove air bubbles by tapping the cuvette

- Or cover the open cuvette end with Parafilm & slowly invert it several times
Fingerprints, liquid droplets, and smudges on the cuvette surface can give false light absorbance readings.

Wipe the outside of the cuvette clean with a Kimwipe.

Always clean the outside, lower portion of the cuvette before taking any readings & only handle the cuvette by the top.
The sample holder is on the top left of the surface of the Spec 20D+

Gently insert the Blank all the way into the sample holder & close the cover
A. SPEC 20D+ CALIBRATION

SUMMARY

• STEP 1: Turn on – Warm up 15 min.
• STEP 2: Set Wavelength
• STEP 3: Set Mode to Transmittance
• STEP 4: Set Transmittance to 0%
• STEP 5: Set Mode to Absorbance
• STEP 6: Prepare and Insert Blank
• STEP 7: Set Absorbance of the Blank to Zero
SET ABSORBANCE OF BLANK TO ZERO

➢ The absorbance adjust knob is the right dial on the front of the instrument

➢ Set absorbance to zero at your desired wavelength with your sample tube in place & the cover closed
B. GENERATE A SPECTROGRAM

- You have now calibrated the Spec 20D+
- Remove the Blank from the sample chamber
- You are now ready to generate data for your Spectrogram!
B. GENERATE A SPECTROGRAM

SUMMARY

• STEP 1: Insert Sample
• STEP 2: Read Absorbance of Sample
Insert Sample

- Keep wavelength set to 610nm
- Gently insert 1 of the blue “sample solution” cuvettes into the sample chamber & close the cover

**Make sure the sample cuvette is clean & bubble-free!**
Read Absorbance of Sample

➢ The Spec 20D+ will begin reading the absorbance as soon as you insert the cuvette into the chamber

➢ Read & record the absorbance of your sample from the digital display \textit{when the number stabilizes}
B. GENERATE A SPECTROGRAM

SUMMARY

• STEP 1: Insert Sample
• STEP 2: Read Absorbance of Sample
• STEP 3: Increase Wavelength
INCREASE WAVELENGTH

- Increase the wavelength by 50 nm
B. GENERATE A SPECTROGRAM

SUMMARY

• STEP 1: Insert Sample
• STEP 2: Read Absorbance of Sample
• STEP 3: Increase Wavelength
• STEP 4: Insert Blank & Readjust Absorbance to 0
Insert the Blank all the way into the sample holder & close the cover.

Then, set absorbance to 0.
B. GENERATE A SPECTROGRAM

SUMMARY

• STEP 1: Insert Sample
• STEP 2: Read Absorbance of Sample
• STEP 3: Increase Wavelength
• STEP 4: Insert Blank & Readjust Absorbance to 0
• STEP 5: Remove Blank & Insert Sample
• STEP 6: Read Absorbance of Sample
Read Absorbance of Sample

- Insert the **same** sample you read at the previous wavelength

- Read & record the absorbance of your sample at the new wavelength **when the reading stabilizes**
B. GENERATE A SPECTROGRAM

SUMMARY

• STEP 1: Insert Sample
• STEP 2: Read Absorbance of Sample
• STEP 3: Increase Wavelength
• STEP 4: Insert Blank & Readjust Absorbance to 0
• STEP 5: Remove Blank & Insert Sample
• STEP 6: Read Absorbance of Sample
• STEP 7: Repeat Steps 3 – 6 to record absorbances of your sample every 50 nm from 610 through 910 nm
B. GENERATE A SPECTROGRAM

- Label axes & plot the absorbance readings \((y)\) at each wavelength \((x)\) on graph paper
- Smoothly connect the data points with a line
- Determine the wavelength of peak absorbance \((\lambda_{\text{max}})\) from the graph

![Spectrogram](image)
C. CREATE A CALIBRATION CURVE

- Now you know the wavelength ($\lambda_{\text{max}}$) at which light is maximally absorbed by your substance of interest (CuSO$_4$)
- You are now ready to create a Calibration Curve!
C. CREATE A CALIBRATION CURVE

SUMMARY

• STEP 1: Note the concentration of the sample you used to generate your Spectrogram & the absorbance at $\lambda_{\text{max}}$ of that sample
C. CREATE A CALIBRATION CURVE

SUMMARY

• STEP 1: Note the concentration of the sample you used to generate your Spectrogram & the absorbance at $\lambda_{\text{max}}$ of that sample

• STEP 2: Set the wavelength on the Spec 20D+ to $\lambda_{\text{max}}$
C. CREATE A CALIBRATION CURVE

SUMMARY

• STEP 1: Note the concentration of the sample you used to generate your Spectrogram & the absorbance at $\lambda_{\text{max}}$ of that sample
• STEP 2: Set the wavelength to $\lambda_{\text{max}}$
• STEP 3: Insert the Blank & set the absorbance to 0
C. CREATE A CALIBRATION CURVE

SUMMARY

- **STEP 1:** Note the concentration of the sample you used to generate your Spectrogram & the absorbance at $\lambda_{\text{max}}$ of that sample
- **STEP 2:** Set the wavelength to $\lambda_{\text{max}}$
- **STEP 3:** Insert the Blank & set the absorbance to 0
- **STEP 4:** Insert a sample with a different concentration & record the absorbance of that sample
C. CREATE A CALIBRATION CURVE

SUMMARY

• STEP 1: Note the concentration of the sample you used to generate your Spectrogram & the absorbance at $\lambda_{\text{max}}$ of that sample
• STEP 2: Set the wavelength to $\lambda_{\text{max}}$
• STEP 3: Insert the Blank & set the absorbance to 0
• STEP 4: Insert a sample with a different concentration & record the absorbance of that sample
• STEP 5: Repeat Steps 3 & 4 with the two remaining samples with different concentrations
C. CREATE A CALIBRATION CURVE

- Label axes & plot the absorbance readings (y) at each sample concentration (x)
- Use a ruler to draw the best fit line
D. DETERMINE THE CONCENTRATION OF AN UNKNOWN SAMPLE

- You are now ready to take & PASS the Post-Test!
  - Obtain a CuSO4 sample of unknown concentration and a posttest form from a Science Learning Center Assistant
  - Fill in answers to the posttest questions and submit it along with your Spectrogram and Calibration Curve graphs.